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Cyclic AMP, Glucocorticoid, and Retinoid Modulation of in Vitro Keratinocyte Growth

CYNTHIA L. MARCELO, PH.D., AND JOHN TOMICH, M.D.

Dermatology Department, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

The hypothesis that an imbalanced cyclic AMP and cyclic GMP ratio was central to the cutaneous expression of psoriasis prompted the design of a series of in vitro experiments. The aim of these studies was to describe the functional effects of increased intracellular cyclic AMP and of drugs therapeutic in psoriasis on epidermal keratinocyte growth. Epidermal basal cells trypsinized from neonatal mouse and adult and neonatal human skin were grown on plastic or on gelled collagen surfaces. These were used to study the effect of cyclic AMP analogues and cholera toxin (an irreversible stimulator of cyclic AMP synthesis) on keratinocyte growth. Greatly increased intracellular cyclic AMP levels, that is, 60-fold to 70-fold, stimulated neonatal mouse keratinocyte proliferation and differentiation; these same doses were cytotoxic to both neonatal and adult human cells. However, modest increases in intracellular cyclic AMP did stimulate adult human keratinocyte proliferation. The glucocorticoid triamcinolone acetonide inhibited neonatal mouse keratinocyte proliferation for approximately 1 week; the cells then became refractory to the triamcinolone acetonide effect. Triamcinolone acetonide did not apparently act through cyclic AMP-mediated events. In fact, this glucocorticoid inhibited cyclic AMP-stimulated epidermal keratinocyte proliferation. Likewise, vitamin A analogues, including the psoriasis therapy drug Ro 10-9359 inhibited neonatal mouse keratinocyte proliferation and specific differentiation events; the retinoids therapeutic in psoriasis apparently did not act via cyclic AMP-mediated events and inhibited cyclic AMP-stimulated functions. Our results indicated that cyclic AMP is a mitogenic signal for epidermal keratinocytes. This cyclic nucleotide may be important in regulating epidermal hyperproliferation. A central role for cyclic AMP in the cutaneous expression of psoriasis, however, is yet to be proven.

In the mid-1970s, a large amount of experimental evidence suggested that cyclic nucleotides, especially cyclic AMP, were involved in the development of hyperproliferative skin lesions [1,2]. At that time, an imbalanced cyclic AMP to cyclic GMP ratio was shown to occur in frozen epidermal tissue taken from both plaque and uninvolved areas of psoriatic individuals [1,3]. These data strongly suggested that the cyclic nucleotide system of the psoriatic epidermis was important in the manifestation of this hyperproliferative skin disease [1–3]. Thus the research had gotten to a point where it became essential for us to find out if cyclic AMP and cyclic GMP had functional importance in the epidermis, although a number of studies using whole skin [4], human skin explant cultures [5], and guinea pig cells [6] reported that high doses of cyclic AMP-elevating agents could inhibit keratinocyte proliferation.

In this report, we will describe the studies that were done in order to understand the role of cyclic AMP and the effect of therapeutic cutaneous drugs on in vitro epidermal keratinocyte function. Most of the data presented and summarized here were obtained using a neonatal mouse primary keratinocyte system developed in these laboratories [7]. Adult human and neonatal human keratinocyte cultures were grown on collagen beds as described by Lui and Karasek [8] and were used for additional cyclic AMP function studies.

MATERIALS AND METHODS

Epidermal Tissue Sources

Neonatal mice were obtained from a BALB/c colony housed in the University of Michigan Medical School Dermatology Department. Adult human epidermis was obtained from volunteers at the University Hospitals Dermatology Outpatient Clinic, following approved human use protocols as defined by the University of Michigan. Neonatal human foreskins were obtained from the University Hospitals Nursery.

Reagents

Medium-199 (M-199, modified with Earle's salts, glutamine), minimum essential medium (MEM, Eagle, modified with Earle's salts, glutamine), McCoy's 5A (Iwakata and Grace modification, glutamine), Vitrogen 100 purified collagen, fetal bovine serum, newborn calf serum, and all tissue-culture reagents were purchased from Flow Laboratories Inc. (Rockville, Md.). Trypsin (2× crystallized), Ficoll 400, collagenase (type 11-5), 8-bromo cyclic AMP, dibutyryl cyclic AMP, all-*trans*-vitamin A (retinoic acid), vitamin A alcohol (retinol, trans), triamcinolone acetonide, and β-cortisol were from Sigma Chemical Co. (St. Louis, Mo.). Thymidine [methyl-^{3}H] ([^{3}H]-TdR) was obtained from New England Nuclear (Boston, Mass.). Cholera toxin was purchased from Schwarz Mann (Orangeburg, N.Y.). Ro 10-9359 trimethylmethoxyphenyl (TMMP) analogue of retinoic acid ethyl ester (etretinate) and 13-*cis*-retinoic acid (Ro 4-3780, isotretinoin) were a gift from Hoffmann-La Roche, Inc. (Nutley, N.J.).

Epidermal Keratinocyte Cultures

Neonatal mouse primary cultures. As previously described [7], trypsinization at 37°C was used to separate the epidermis from the dermis. After centrifugation on a 15 to 20 percent Ficoll gradient, viable basal cells were plated in M-199 plus 10 to 13% FBS containing antibodies and antibiotics. Then 2×10^5 cells were plated per cubic centimeter of plastic growth surface. The cultures were grown in 5% CO_2 at 32°C. The medium was changed every second day.

Adult human primary cultures. Epidermal strips obtained from normal human volunteers were floated on 0.2% trypsin in PBS containing 0.1% glucose at 37°C for 30 minutes. Keratinocytes from any small pieces of dermis and from the epidermis were then grown as described by Lui and Karasek [8]. The growth-surface area was ammonium hydroxide-fume cross-linked rabbit dermis collagen (mostly type I) or Vitrogen (calf tail collagen) cross-linked by adjusting the collagen suspension to physiologic pH and ionic strength and gelling at 37°C.

Neonatal human primary cultures. Neonatal human keratinocytes were isolated from foreskins that were dissociated into epidermal and dermal components by floatation, dermis down, on 0.2% trypsin in PBS containing 0.1% glucose overnight at room temperature. The procedures were then identical to those used for the adult human cultures.

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Reprint requests to: Dr. Cynthia Marcelo, Box 056, Dermatology, Kresge I, R-6558, Ann Arbor, Michigan 48109.

Abbreviations:
PBS: phosphate-buffered saline
FBS: fetal bovine serum
HETE: hydroxyeicosatetraenoic acid
[^{3}H]-TdR: titrated thymidine
M-199: Medium-199
MEM: minimum essential medium
SEM: standard error of the mean
TMMP: trimethylmethoxyphenyl

TABLE I. *Neonatal mouse cultures*

8-Bromo cAMP, 10^{-3} to 10^{-5} M	Increases proliferation
Cholera toxin, 1 μg to 50 ng/ml	Increases proliferation
Dibutyryl cAMP, 10^{-3} to 10^{-5} M	Increases proliferation
8-Bromo cAMP, 10^{-3} M	Increases cAMP in cells 66×
Cholera toxin, 1 μg/ml	Increases cAMP in cells 8.6×
Cholera toxin, 50 ng/ml	Increases cAMP in cells 3.5×
8-Bromo cAMP, 5×10^{-4} M	Increases differentiation

Source: From Marcelo et al. [9]: Tong and Marcelo (unpublished) [10].

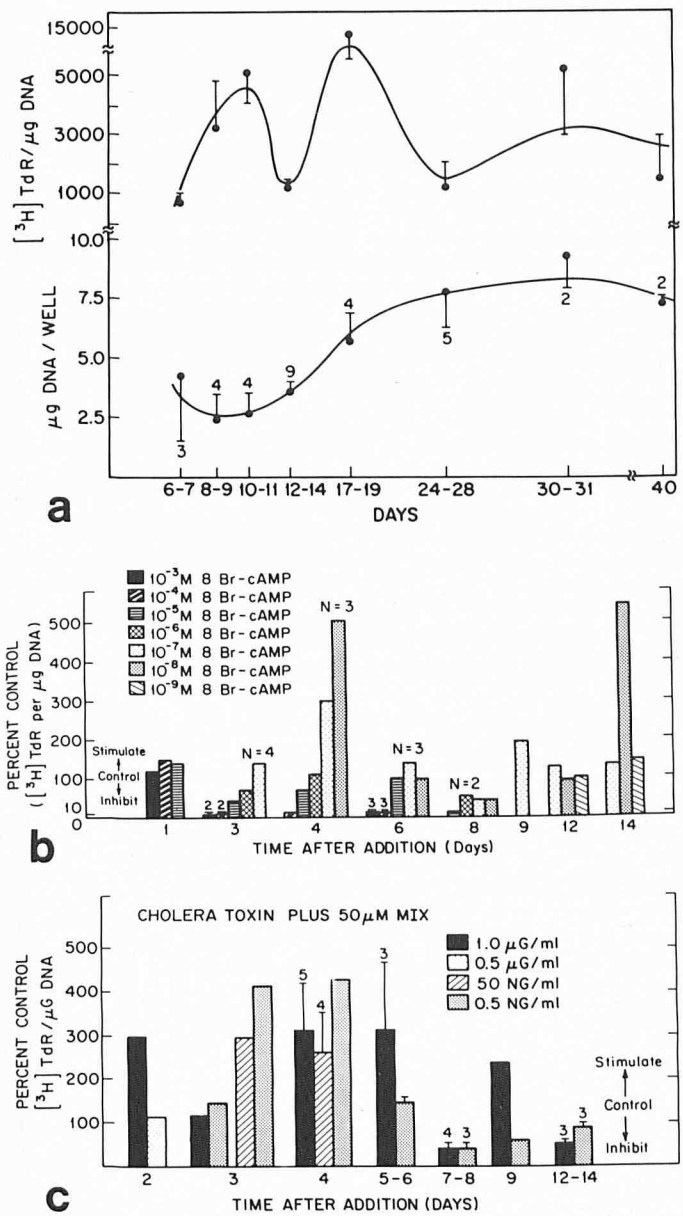

FIG 1. Effect of cyclic AMP-elevating agents on adult human keratinocyte proliferation. *a*, Growth curve of adult human keratinocytes on collagen gels using technology described by Lui and Karasek [8] over 40 days. Data are presented as mean ± SEM; the *n* values are given. *b*, Effect of seven concentrations of the cyclic AMP analogue 8-bromo cAMP on adult human keratinocyte incorporation of [^{3}H]-TdR into DNA. There were no changes in the ^{3}H label in the acid-soluble pool unless obvious cytotoxicity was seen, i.e., loss of monolayers or sharp decreases in total DNA or protein. *c*, Effect of cholera toxin, an irreversible stimulator of adenylate cyclase, on the incorporation of [^{3}H]-TdR into DNA. MIX is isomethyl-butyl xanthine, an inhibitor of the cyclic AMP-degrading enzyme phosphodiesterase. Selected autoradiographic studies of control and treated cultures showed the same results as the biochemical data.

Fibroblast cultures. Adult human fibroblasts were isolated using 0.1% collagenase in PBS digestion of small pieces of dermis that resulted from the keratome biopsy procedure. Then 1×10^5 cells per 60-mm Petri dish were plated in MEM growth medium and the cells were cultured at 37°C.

[^{3}H]-TdR labeling and autoradiography. Keratinocyte and fibroblast cultures were terminally pulse labeled for 6 hours with 1 μCi/ml of [^{3}H]-TdR, as previously described [7]. Autoradiography was done as previously reported [7].

RESULTS

Effect of Cyclic AMP-Elevating Agents

Neonatal mouse keratinocytes. As summarized in Table I, when cyclic AMP analogues and cholera toxin, an irreversible stimulator of adenylate cyclase (ATP → cyclic AMP), were added to the primary neonatal mouse cultures, an extensive stimulation of [^{3}H]-TdR incorporation into DNA was observed, as was an increase in the percent labeled nuclei per culture [9,10]. The addition of the high dose of 10^{-3} M 8-bromo cyclic AMP as well as the low dose of 50 ng/ml cholera toxin increased the cellular levels of cyclic AMP 66-fold and 3.5-fold, respectively. Proliferation was assessed by use of the [^{3}H]-TdR labeling technique, which is a reliable indicator of proliferation in these cultures [11]. Increases in cellular cyclic AMP levels also stimulated the amount and synthesis of keratins and keratohyalin granule-related and cell-envelope proteins [12].

Adult human keratinocytes. As presented in Fig. 1*a*, adult human keratinocytes grown on collagen gels took approximately 10 to 14 days to form a stable, stratifying culture. The cultures grew easily for 40 to 50 days under these conditions. As seen in Fig. 1*b*, 8-bromo cyclic AMP at high doses, that is 10^{-3} to 10^{-5} M, was cytotoxic. The monolayers were destroyed so that there were no data points for these doses at the later time points. It did appear that 8-bromo cyclic AMP at the lowest doses stimulated keratinocyte incorporation of [^{3}H]-TdR into DNA; however, this effect lasted for only a short time (2–3 days). Cholera toxin, which causes a twofold to fivefold increase in cellular cylic AMP levels (a modest increase of cyclic AMP), stimulated adult human keratinocyte proliferation (see Fig. 1*c*), but as seen with 8-bromo cyclic AMP, the effect lasted for only a short time (approximately 1 week).

Neonatal human keratinocytes. In Table II, the results of a study using neonatal human keratinocyte cultures grown on collagen gels are presented. The study covered a 15-day time period, and 8 doses of 8-bromo cyclic AMP were tested. 8-Bromo cyclic AMP at 10^{-3} and 10^{-4} M were cytotoxic to the cultures. No stimulation of [^{3}H]-TdR incorporation into DNA by this cyclic AMP analogue was observed. Addition of cholera toxin did not stimulate neonatal human keratinocyte proliferation (data not presented). As shown in Fig. 2, 10^{-3} (not shown) and 10^{-4} M (Fig. 2*b*) 8-bromo cyclic AMP destroyed the cultures; at lower doses, the morphology of the culture was identical to that of the control (Fig. 2*a*).

TABLE II. *Effect of 8-bromo cyclic AMP on neonatal human keratinocyte incorporation of [^{3}H]-TdR into DNA*

Concentration	Days grown in 8-bromo cyclic AMP (percent control)[b]				
	3	6	9	12	15
10^{-3} M	4%	4%	1%	1%	5%
10^{-4} M	11%	4%	1%	1%	6%
10^{-5} M[a]	70%	129%	61%	41%	118%
10^{-6} M	76%	90%	68%	77%	81%
10^{-7} M	65%	88%	86%	96%	113%
10^{-8} M	62%	102%	89%	88%	116%
10^{-9} M	78%	60%	106%	92%	94%
10^{-10} M	75%	70%	96%	60%	76%
None	100%	100%	100%	100%	100%

[a] Except for the 10^{-3} and 10^{-4} M doses, no change in the acid-soluble ^{3}H-labeled pools occurred.

[b] All cultures were 3–4 day monolayers when the 8-bromo cyclic AMP was added.

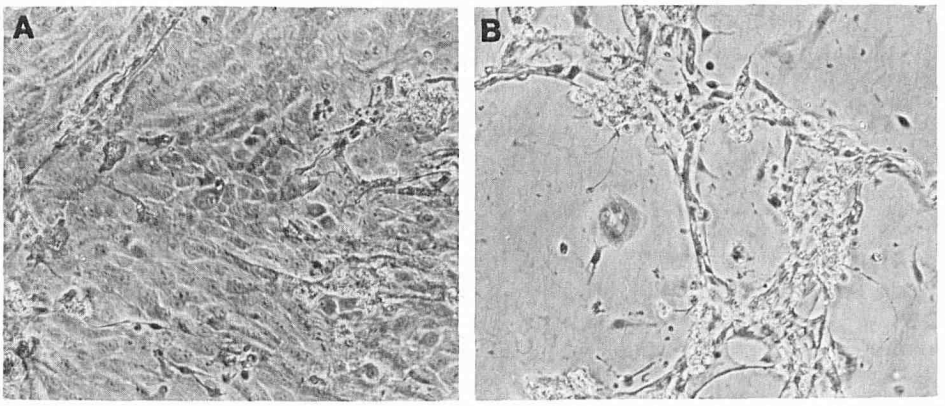

FIG 2. Phase micrographs of control and 8-bromo cyclic AMP-treated neonatal human keratinocytes. The 8-bromo cyclic AMP was added to complete monolayer cultures (4–5 days old). *a*, Control. *b*, 10^{-4} *M* 8-bromo cyclic AMP (reduced from ×300).

TABLE III. *Effect of 8-bromo cyclic AMP and cholera toxin on adult human fibroblasts*

	DNA (μg)	DNA (cpm/μg)	Percent change
Control	13.33 ± 0.29	732	—
8-Bromo cyclic AMP:			
10^{-3} *M*	6.40 ± 1.4	130 ± 27	82% ↓
10^{-4} *M*	7.01 ± 0	308 ± 38	58% ↓
10^{-5} *M*	8.17 ± 0.08	722	2% ↓
10^{-6} *M*	13.00 ± 0.8	477 ± 22	35% ↓
10^{-7} *M*	8.96 ± 4.16	806 ± 100	10% ↑
10^{-8} *M*	9.94 ± 1.69	396 ± 50	46% ↓
1 μg Cholera toxin + 50 μ*M* MIX	8.87 ± .210	1300 ± 300	90% ↑
0.50 ng Cholera toxin + 50 μ*M* MIX	13.20 ± 6.0	1403 ± 600	92% ↑

Note: Drugs added to $D = 20$ quiescent cultures. [^{3}H]-TdR assay done on day 6 after addition of the 8-bromo cyclic AMP and toxin.

Adult human fibroblasts. In Table III, the results of a study of the effect of 8-bromo cyclic AMP and cholera toxin on adult human fibroblast cultures are presented. The 8-bromo analogue of cyclic AMP inhibited fibroblast proliferation, while cholera toxin might be considered slightly mitogenic. The fibroblast response to cyclic AMP-elevating agents differed from that observed in the human keratinocyte culture studies (Figs. 1,2; Table II).

Triamcinolone Acetonide

As presented in Fig. 3*a*, triamcinolone acetonide (*ta*) inhibited neonatal mouse keratinocyte proliferation. Triamcinolone acetonide was 1 to 2 log units (100-fold) more potent than 11β-cortisol, another therapeutically active glucocorticoid.

As depicted in Fig. 3*b*, the inhibitory effect of triamcinolone acetonide was transitory. In this series of experiments, 10^{-8} *M* triamcinolone acetonide was used; the inhibitory effect was not observed after day 8 of growth in the drug.

Effect of Triamcinolone Acetonide on Cyclic AMP Stimulated Proliferation

In Fig. 4, the results of experiments studying the effect of three doses of triamcinolone acetonide and three doses of 8-bromo cyclic AMP, in all nine possible combinations, on neonatal mouse keratinocyte culture proliferation are presented. As seen in Fig. 4*a*, 10^{-10}, 10^{-9}, and 10^{-8} *M* triamcinolone acetonide inhibited cyclic AMP-stimulated proliferation at this time point ($N = 2$); 10^{-5} *M* 8-bromo cyclic AMP is not mitogenic the second day after addition [10]. After 3 days (Fig. 4*b*), the triamcinolone acetonide effect was still observed, and the lowest dose of this glucocorticoid (10^{-10} *M*) was the most effective inhibitor ($N = 4$). After 7 days, the inhibitory effect of triamcinolone acetonide on cyclic AMP-stimulated proliferation was much lessened ($N = 2$; Fig. 4*c*).

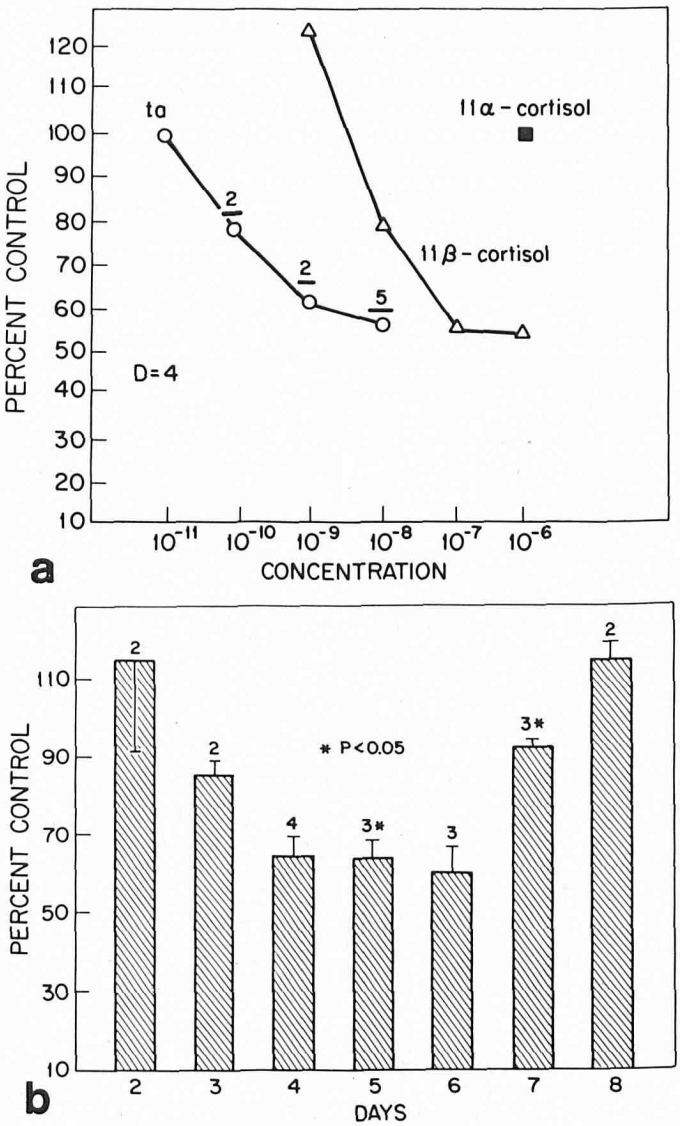

FIG 3. Effect of triamcinolone acetonide on neonatal mouse keratinocyte proliferation. *a*, Dose response of triamcinolone acetonide (*ta*; ○) and 11β-cortisol (△) on [^{3}H]-TdR incorporation into DNA. 11α-Cortisol (■) is an inactive isomer of the corticosteroid 11β-cortisol. Control = 100%; day (D) 4 after addition; mean ± SEM. *b*, Effect of 10^{-8} *M* triamcinolone acetonide on [3H]-TdR incorporation into DNA. Mean ± SEM; and the students *t* test was used to assess the significance of the inhibitory effect.

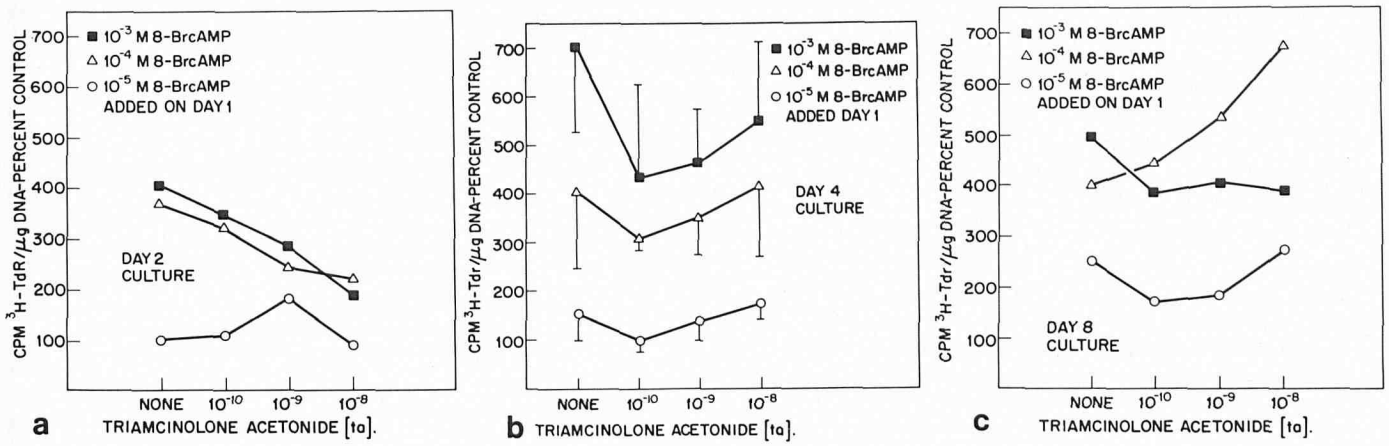

FIG 4. Effect of triamcinolone acetonide on 8-bromo cyclic AMP-stimulated neonatal mouse cultures. All the agents were simultaneously added to the cultures 1 day after plating. All nine possible combinations of triamcinolone acetonide (10^{-8}, 10^{-9}, and 10^{-10} *M*) and of 8-bromo cyclic AMP (10^{-3}, 10^{-4}, and 10^{-5} *M*) were tested. *a*, On day 1 after addition, the inhibition by triamcinolone acetonide of 8-bromo cyclic AMP-stimulated proliferation was dose-dependent. *b*, After 3 days, triamicinolone acetonide was inhibitory, although the lowest dose, 10^{-10} *M*, was most effective. *c*, After 7 days, triamcinolone acetonide was not as effective an inhibitor. Neither drug changed the amount of ^{3}H label in the acid-soluble pool. Autoradiography of selected doses and time periods showed the same results.

TABLE IV. *Vitamin A analogues*

Aromatic retinoid (RO 10-9359) (*μM* to *nM*)	Inhibits proliferation
	Inhibits differentiation
13-*cis*-Retinoic acid	Inhibits proliferation
	Inhibits differentiation
All-*trans*-retinoic acid	Inhibits proliferation
	Inhibits differentiation
Retinol	Inhibits proliferation
	Inhibits differentiation
Arotinoid[a] (RO 13-6298) (*μM* to p*M*)	Inhibits differentiation
	Inhibits proliferation

[a] Retinoids can inhibit cAMP-induced stimulation.
Source: Stadler et al., unpublished data.

Vitamin A Analogues

The effect of a group of vitamin A analogues is summarized in Table IV [13; Stadler, unpublished data]. In studies using the neonatal mouse keratinocyte culture system, the aromatic retinoid Ro 10-9359 (Psoriasis drug; etretinate), 13-*cis*-retinoic acid (isotretinoin), all-*trans*-retinoic acid, and retinol inhibited epidermal keratinocyte proliferation (dose *μM* to p*M*). There was a difference among the retinoids as to potency. Some retinoids, such as retinol, were very powerful inhibitors, while others, such as Ro 10-9359, were much less inhibitory [Marcelo and Madison, Arch Dermatol Res (in press)]. The Ro 10-9359, 13-*cis*- and all-*trans*-retinoic acids inhibited keratin polypeptide synthesis and cell-envelope formation by these keratinocytes [13; Marcelo and Madison, Arch Dermatol Res (in press)]. The aromatic Ro 10-9359 retinoid and a new experimental retinoid called Arotinoid (Ro 13-6298), presently being studied in our laboratories by Dr. R. Stadler, increased the amounts of keratohyalin granule-related proteins in these keratinocyte cultures [Stadler, unpublished results; Marcelo and Madison, Arch Dermatol Res (in press)].

Another interesting result recently observed by us is that both the aromatic (Ro 10-9359) and Arotinoid (Ro 13-6298) drugs can inhibit cyclic AMP-stimulated proliferation and cholera toxin-induced cyclic AMP synthesis (Stadler, unpublished data).

DISCUSSION

Conceptually, these studies flowed from the hypothesis that cyclic AMP was decreased and that cyclic GMP was increased in psoriatic lesions and that this imbalance was central to the disease state. This premise allowed the following predictions to be made:

Elevation of intracellular cyclic AMP would inhibit in vitro epidermal keratinocyte proliferation and would stimulate, or permit, the expression of differentiation.

Increased cyclic GMP levels would stimulate epidermal keratinocyte proliferation.

Agents therapeutic for psoriasis would stimulate in vitro keratinocyte cyclic AMP levels and would inhibit keratinocyte proliferation.

As usually happens, the working hypothesis failed to predict the complexity of the effect of cyclic AMP-elevating agents and therapeutic drugs on in vitro epidermal keratinocyte function.

The cyclic AMP analogue and cholera toxin studies presented in this report showed that isolated neonatal mouse and adult human keratinocytes grown in primary culture were stimulated to proliferate by intracellular increases in cyclic AMP. Cyclic GMP did not stimulate keratinocyte proliferation [7,12]. Large increases in cyclic AMP were cytotoxic to the adult human keratinocytes in culture, as has been reported by others [4–6]. However, modest increases in intracellular cyclic AMP mediated by cholera toxin activation of the cyclic AMP-synthesizing enzyme adenylate cyclase increased the proliferation of the adult human cells for a defined period of time [14]. Thus it seems that primary neonatal mouse keratinocyte cultures can be used to predict what would happen in the adult human system.

The results of our in vitro studies are in agreement with similar in vivo studies. Murray et al. [15] and Liu and Galloway [16] have reported that cholera toxin increases epidermal cyclic AMP levels in adult mice and rabbits. Kuroki [17] has reported that cholera toxin induces synchronous cell division, causing hyperplasia in the epidermis of adult mice, rats, and hamsters, while Jumblatt, Fogle, and Neufeld [18] have reported enhanced epithelial wound healing in in vivo rabbit cornea. Thus cyclic AMP elevation has been demonstrated to stimulate in vivo epidermal proliferation.

However, for some as yet unexplained reason, cyclic AMP elevation did not stimulate isolated neonatal human keratino-

cyte proliferation on collagen gels. The simplest explanation of this observation is that the human epidermal keratinocyte in the neonatal state is not responsive to cyclic AMP. It is possible that the neonatal mouse keratinocyte is initially also unresponsive, but because of its capacity for very rapid ontogenic development, adult (mouse) keratinocyte response to cyclic AMP by cells taken from neonates is seen in vitro. It has recently been suggested by Okada, Kitano, and Ichihara [19] that cyclic AMP responsiveness is dependent on cell plating density and the rate of keratinocyte proliferation. This observation does not pertain to these systems, since both the neonatal mouse and adult and neonatal epidermal keratinocytes were plated at maximum density (complete monolayer by day 2), did not demonstrate contact inhibition of proliferation [7], and especially in the case of adult cells, did not respond to cyclic AMP at any time during the 40-day time period that was studied.

Triamcinolone acetonide, a potent therapeutic psoriasis drug [20], acted as predicted: it decreased epidermal keratinocyte proliferation. Triamcinolone acetonide, however, did not act by increasing keratinocyte cyclic AMP levels nor by any other cyclic AMP-dependent mechanisms (Marcelo, unpublished data); moreover, this glucocorticoid inhibited cyclic AMP-stimulated proliferation.

The vitamin A analogues, the retinoids, are another series of drugs that affect epidermal keratinocyte function. The two psoriasis therapeutic drugs Ro 10-9359 and Ro 13-6298 inhibited keratinocyte proliferation [13; Stadler, unpublished]. Again, as seen with triamcinolone acetonide, Ro 10-9359 and Ro 13-6298 did not act by changing cellular cyclic AMP levels, but they did inhibit cyclic AMP-stimulated proliferation and cholera toxin-induced cyclic AMP synthesis.

Thus, since the early 1970s, our in vitro systems have allowed us to understand and to rethink our theories concerning cyclic nucleotides and psoriasis. Our results [3,10,12,13] and those of a number of other investigators [14–19] suggest that cyclic AMP is probably an important modulator of epidermal keratinocyte function and may be increased in hyperproliferative skin disorders. A central role for cyclic AMP or cyclic GMP in the onset of psoriasis is at present not proven.

Other theories have replaced the cyclic AMP-cyclic GMP hypothesis in the effort to understand and control the psoriatic condition. These working hypotheses focus on the role of arachidonic acid products, specifically the HETEs and leukotrienes [21], as well as such intracellular modulators as calmodulin. In the future, the in vitro systems described in this report will be used to study the role of these modulators on epidermal keratinocyte function and to help us understand, and again to rethink, our theories concerning the control of epidermal keratinocyte function.

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